Mycotoxins in Indoor Environments

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Abstract
Exposure to mycotoxins produced by toxigenic molds growing in damp indoor spaces has been difficult to assess. Monitoring methods limit the characterization of inhalation exposure of any bioaerosol, especially that of mycotoxins. Biomarkers promise better ability to determine mycotoxin exposures 1.) through direct measures of toxins and their products in human tissues, 2.) through immunochemical methods, and 3.) measures of effect through novel approaches, e.g., proteomics or genomics. This paper summarizes both the problems inherent in measuring exposures and some of the promising methods that could help to resolve the current impasse.

Introduction

Reports of illness among occupants of damp buildings have become a concern, especially with respect to both fungi and bacteria that colonize damp spaces. While much illness can be explained as allergic sensitization, a number of complaints involving the immune, respiratory and nervous system have implicated mycotoxins. Epidemiological studies have been able to relate building "dampness" as the lowest common denominator among studies that differ in exposure parameters to asthma and other allergic respiratory disease. Such relationships have not been clearly established for immune and nervous system effects due to a small number of studies, small statistical power of existing studies, and difficulty in assessing agents such as mycotoxins.

Buildings become moist because of leaks in plumbing, roofs, walls, windows, or foundations, through flooding, or due to condensation in areas that lack sufficient ventilation. Certain toxigenic fungi, such as species of *Aspergillus, Penicillium, Stachybotrys, Memnoniella,* and *Chaetomium* grow frequently in damp indoor environments under conditions in which they can produce mycotoxins (1, 2). The various fungi and other microorganisms such as bacteria, yeasts, and protozoa form an ecosystem that changes over time as moisture and nutrient levels and activity of the microorganisms change. Competition for available ecological niches also can increase production of mycotoxins.

Mycotoxins are well-known agents of disease in animals and humans who consume them in foods in sufficient amounts. Inhalation of toxins associated with grain dusts and other organic dusts are known to be a problem for workers who have developed pulmonary mycotoxicoses (including, but not limited to organic dust toxic syndrome or ODTS). Establishing that illnesses seen in workers and other occupants of damp indoor environments are caused by inhalation of mycotoxins has been more difficult. This paper examines reasons

why this has occurred and explores means of determining better estimates of exposure to indoor (mold and) mycotoxins.

Methods

Information regarding health effects from exposure to mycotoxins produced by toxigenic molds commonly found in damp indoor spaces is reviewed. Evidence for considering other exposure agents besides spores of toxigenic fungi is examined, and the lack of correlation of toxins with disease in epidemiological studies is explored. Methods for identification of mycotoxins in environmental media and within human tissues are discussed. Biomarkers and broadening the concept to markers of toxic effect are considered.

Discussion

Symptoms Reported from Occupants of Moldy Buildings

The most frequent complaints of occupants of moldy buildings are of cough, rhinosinusitis and worsening of asthma, and increased susceptibility to respiratory and other infections. Such infections last longer than "normal," recur and are often recalcitrant to treatment as occurs in immune-compromised patients. Complaints of extreme fatigue, headache, memory problems, difficulty in concentrating or thinking clearly, numbness and other nervous system effects are also not rare. Complaints overlap toxic, allergenic and irritative symptoms, and are difficult to assign to specific agents found in buildings (3).

Molds that grow in damp buildings, and a number of bacteria, especially *Streptomyces* species, have been implicated in studies addressing signs of allergy, such as increase in inflammatory markers, or increased immunoglobin titers (3). Allergic reaction to molds can explain such signs and symptoms in atopic people, while those who are not atopic often have illnesses (3, 4), that are likely due to other causes such as toxicity. Allergy also does not explain increased susceptibility to infection and nervous system effects. These are in keeping with mycotoxicoses in herd animals consuming moldy feed.

Problems with Measuring Mycotoxin Exposure Indoors

Inhalation has generally been considered the route of exposure from indoor mold contamination. Because molds such as *Aspergillus* and *Penicillium* colonize new habitats by casting their spores into the air, inhalation of airborne spores has been thought to be the primary means of mycotoxin exposure in damp buildings. However, mycotoxins are not found only in and on spores (1, 5). They are also found in hyphae and hyphal and spore fragments, which are considerably smaller than whole spores or conidia. Mycotoxins are secreted into the substrate on and in which molds grow, and are found on dust from substrate (1, 5, 6, 7, 8) as well as in small airborne particles generated directly by the organisms (6, 10). Settled

carpet dusts from damp buildings contain mycotoxins (7) as do dusts from ventilation systems (8, 9).

A recent study of aerosolization of fungal propagules using a newly designed aerosolization chamber, determined that fragments of molds are released from infested ceiling tiles in much greater numbers than spores. Depending on fungal species, velocity of air past contaminated surfaces, texture of the surface, and amount of vibration, concentration of small particles released may be 320 times greater than spore concentration (6). These particles are of considerably smaller size than spores (1.6 µ average for fragments, compared to 2-3.5 µ for *Aspergillus versicolor* spores, and 4-7 µ for *Cladosporium cladosporoides* spores) (6). Air velocities as low as 0.3 meters/second caused spores and small particles to be released (11).

Inhalation of a mixture of spores, fragments, fungal propagules and dust represents a much greater potential for exposing occupants to mycotoxins than inhalation of spores alone. Smaller particles, with their large aggregate surface area, can adsorb more mycotoxins than the same mass of larger spores. Small particles are more easily inhaled and deposited in airways and the lung, where toxins can cause damage locally, or be absorbed into the bloodstream and lymphatic circulation and distributed to target organs. Measuring toxin concentration of settled or airborne dust could more realistically estimate mycotoxin exposure. However, airborne and settled dust samples will reflect different spectra of fungi because of settling characteristics and other factors, so that using both is probably necessary (12). Accurate assessment of exposure is essential for establishing links with mycotoxicoses.

Studies that tried to relate symptoms with spore levels from air samples have found little correlation with incidence or severity. Researchers often conclude that no exposure occurred. Allergic symptoms suffered by atopic persons were attributed to mold exposure, while similar symptoms suffered by non-atopic individuals could not be explained (3), and were dismissed.

Problems with Determining Mold Exposure

Problems inherent to characterization of spore concentrations make correlation with symptoms of occupants difficult (13). Health complaints often predate building investigations, so researchers have to attempt to re-construct episodic past exposures. Non-viable or viable sampling procedures, usually in only a few locations in a building, are performed for short periods of time. Samples are dawn by pumps at a few liters per minute for a 5- or 10- minute period. Spores captured for non-viable sampling are analyzed via light microscopy, and only generic identification can be performed. Closely related species such as *Aspergillus* and *Penicillium* spores cannot be distinguished. Identification to the species level of any spore from these genera requires viable sampling. To capture spores alive, volumes of air are drawn onto liquid or semi-solid growth

media that contain selective nutrients. Sampling times are limited on agar by a need to have spores dilute enough to be separated on the surface so that they can be subcultured and identified. In either case the process of sampling captures only a few moments in the life of airborne spores.

Spore production is episodic and unpredictable, and periodic taking of air samples may miss concentrations of spores that occur with blooms, and overcount more buoyant spores that are likely to remain airborne longer. Such periodic sampling may also miss occupant activities that re-mobilize spores into the air. Whether or not spores that are captured are actually enumerated depends on the skill of the mycologist/microscopist in identifying visible spores in non-viable samples, and on a number of limitations imposed by sampling technology, and the biology of the organisms. The choice of growth medium may not support the growth of all species of spores captured, since different species have different growth requirements. Spores impacting the sides of the sampler may be damaged and not grow. Spores may be driven below the surface of the growth medium, and may not grow. Molds that can produce mycotoxins can inhibit growth of competitors which would not be counted even though they may be impacting occupants. Representative sampling of what occupants breathe during the day is severely compromised by these inherent difficulties.

Air sampling for spores, regardless of the analytical technique used for counting and identification, underestimates exposure to microbes. Since toxins are isolated from captured samples, exposure to toxins is also underestimated. Dust sampling for spores gives a different spectrum of microbes, and has some usefulness for assessing contamination that has occurred over time, but it still underestimates genera that are buoyant, and even if all components are assayed, will still miss toxic content.

Extraction of mycotoxins from captured mold spores has been assumed to be a way of measuring mycotoxin exposure in most studies. Usually the amount of toxins measured is so small as to be below threshold for toxic effect. Because of the problems inherent in capturing representative samples of spores from which exposure can be determined, and because toxins in smaller particles that are more likely carriers of mycotoxins are not characterized, attempts to determine toxic cause of human disease indoors have been largely unsuccessful.

Biomarkers of exposure

The whole problem of exposure assessment of mycotoxins may be more usefully addressed through biomarkers. However, since people are exposed to certain mycotoxins in food as well through inhalation, estimates of exposure indoors must take ingestion exposure into account. A number of biomarker techniques have been developed.

A few mycotoxins can be detected directly in tissues. Ochratoxin can be measured directly in blood and urine and has been used to determine exposure from both ingestion and inhalation. Gliotoxin, produced by *Aspergillus fumigatus,* a mold that is responsible for most cases of invasive aspergillosis, is a strong immune suppressor, and is thought to facilitate the invasion of lung tissue by this mold. A liquid chromatography-tandem mass spectroscopy (LS-MS-MS) assay measured gliotoxin in the lungs and sera of mice infected experimentally with *A. fumigatus*, and in patients with this disease (14). Danish researchers have developed a standardized LC-UV-MS micro-scale method for screening fungal metabolites and mycotoxins from culture extracts, and report that they have been able to detect more than 400 fungal metabolites, including penitrems and macro-cyclic trichothecenes (15). However, it is not yet known whether these can be detected in human sera or other tissues. Recent work on macro-cyclic trichothecene–producing *Stachybotrys chartarum* isolated a protein (stachylysin) that could be a marker for exposure, as well as account for an effect (bleeding from respiratory membranes) (16). Aflatoxin adducts, formed when these mycotoxins interact with DNA, RNA or protein, can be detected in sera and urine of those exposed through a variety of methods. However, DNA-adducts result from DNA-repair, and individual variation in ability to repair affected DNA may not reflect true exposure or true effect.

Detection of toxins using immuno-chemical methods has been well-addressed for many environmental sampling media, especially for food products and livestock. For instance specific monoclonal antibodies against aflatoxins, ochratoxin A, zearalenone, diacetoxyscirpenol and T-2 toxin, have been prepared by various researchers through the application of hybridoma technology to mycotoxins (17). Nanogram-range concentrations in milk, butter, maize, peanuts, peanut butter and porcine kidneys have been detected through the use of enzyme-linked or radio-immunoassay or immunoaffinity chromatography. Assays for other toxins, including aflatoxin M1, 3-acetyl deoxynivalenol fusarenon X and roridin A have been developed by another laboratory (18, 19. 20). Monoclonal antibodies have been developed that are capable of detecting fumitremorgin B, produced by *Aspergillus fumigatus,* in rice, buckwheat and corn in the 10 to 60 ng/g range (21). Satratoxin G and related satratoxins can be detected in environmental samples through an enzyme-linked immunosorbent assay in 100 pg/ml concentrations (22). Detection of mycotoxins in an exposure medium such as food, or in an air sample can be used to estimate nominal exposure and are useful for risk assessment. Further development of methods that can measure mycotoxins in human sera and tissues would enhance the ability to use such techniques in exposed humans and would eliminate some of the uncertainty from extrapolation of exposure from media concentrations, and could be of clinical relevance.

Monoclonal antibodies against aflatoxin-B_1-lysine adducts have been used to measure aflatoxin-albumin adducts in human serum collected from residents in areas at high risk for liver cancer (23). A recent study of Gambian children

discovered that IgA antibodies in saliva were reduced in areas of West Africa with high infection-related mortality. Reduction of antibodies (and immune defense) may occur through aflatoxin exposure (24).

Investigators of an outbreak of lung problems in workers repeatedly exposed to fungi in a water damaged building (25), used an antibody assay for the macrocyclic mycotoxin Roridin A (19, 20). One subject among eight developed elevated Immunoglobin G (IgG) antibodies against Roridin-hemisuccinate Human Serum Albumin (R-H BSA), indicating exposure. However, others thought to have been exposed did not develop this immune response (25). The environmental portion of this investigation focused on spores captured in the building, and did not consider other airborne sources of mycotoxin exposure.

Satratoxin-hemisuccinate Bovine Serum Albumin (S-H BSA) has also been prepared against the potent toxic macrocyclic trichothecene produced by *Stachybotrys chartarum* (26). Immunoglobin G (IgG) antibodies against satratoxin H were measured among patients exposed to a mixture of molds who tested positive for antibodies against *Stachybotrys chartarum* exposure. Both exposure to the mold and exposure to the toxin were quantified through serum antibody tests (27). A follow-up study examined these patients for damage that could account for neuropsychological symptoms and peripheral neuropathies such as numbness, tingling and muscle weakness in the extremities (28). Patients with documented, measured exposure to molds had elevated titers of antibodies (immunoglobulin A, immunoglobin A and immunoglobin M, and IgG) to neural-specific antigens, such as myelin basic protein, myelin-associated glycoprotein, ganglioside GM1, sulfatide, myelin oligodendrocyte glycoprotein, alpha-B-crystallin, chondroitin sulfate, tubulin, and neurofilament, which are markers of neural damage. They also suffered neuropsychological disorders and peripheral neuropathies, as determined by tests.

This series of studies indicates that immunochemical methods may be useful in finding the nexus between exposure to mycotoxins and related disease. These studies (26, 27, 28) also point in the direction of a broader concept of biomarker, which is to include markers of effect. The development of techniques such as genomics and proteomics has made it possible to develop profiles of gene expression that could be related to toxic exposures. A number of the more potent mycotoxins produced by molds commonly found in moisture-damaged buildings are potent inhibitors of protein synthesis. Measurement of changes in protein profile can indicate exposure to specific individual or multiple toxins. Proteomics has been used to study the complex nephrotoxicity of OTA. Microarrays were used to assess OTA-specific profiles of expression involved in DNA damage and apoptosis, response to oxidative stress and inflammatory reactions (29).

Aflatoxins produced most often by *Aspergillus flavus* and *Aspergillus parasiticus,* and sterigmatocystin, produced by *Aspergillus versicolor*, are also potent

inhibitors of protein synthesis. The trichothecenes are among the most potent inhibitors of protein synthesis known. Macrocyclic trichothecenes, such as the satratoxins, verrucarins B and J, Roridin A produced by *Stachybotrys chartarum* and Ochratoxin A, produced by both Aspergillus and Penicillium species, are all potent inhibitors of protein synthesis (30).

A number of effects that result from inhibition of protein synthesis can be related to symptoms of occupants of damp buildings (Table 1). Key among symptoms that are reported frequently are those that affect the immune system (induction of autoimmunity, greater susceptibility to infectious disease), neuropsychological deficits, headache, nausea, and deficits in memory and concentration. Protein synthesis is important for antibody and cytokine production and other markers of immunologic effect. Learning and memory depend on protein synthesis, and depression of protein synthesis in the brain could account for frequent reports of memory and learning deficits in mold-exposed patients. The use of proteomics to detect changes in protein patterns as the result of exposure to protein synthesis–inhibiting mycotoxins is another tool that could bring more linkage between exposure to mycotoxins indoors and effects suffered by occupants of moldy buildings.

Use of measures that assess total toxicity can be used in conjunction with mycotoxin assays and environmental contamination measures to establish relationships between toxic exposure and disease. For instance, total toxicity of captured microbial material (spores, fragments and toxin containing particles) can be determined through toxicity assays such as the methylthiazoltetrazolium (MTT)-cleavage test for cellular toxicity. Determination of cytotoxicity of the entirety of environmental samples is a first key step in determining toxic influence on disease from exposure to damp environments (31). Such assays have already been used in clinical investigations, although mycotoxin assessment was limited to toxicity of captured spores (32).

Conclusion

Epidemiological studies, with few exceptions, have been unsuccessful in associating mycotoxin exposure from molds growing indoors. The reason for this has been the lack of means to determine toxic concentrations in inhaled materials. Attention has focused on extracting toxins from spore samples. Yet there is no means of measuring actual exposure to spores through the airborne route. Inhaled particles of fungal or substrate origin that contain mycotoxins are more likely to be agents of exposure, since they have a larger aggregate surface area to which mycotoxins can adsorb, and they are more likely to be inhaled and deposited into the lung.

Such particles, like spores, are likely to have more than toxic effects. For instance, they may also be allergenic or irritating to mucous membranes. Symptoms of allergy and irritation can complicate the presentation of disease

since they overlap those caused by toxicity. However, biomarkers such as those described in this paper may make toxic exposure determination more comprehensive. However, representative assessment of mold species growing in damp indoor environments remains tentative, and standardized methods for characterizing air concentrations are essential, and underlie determination of toxic exposure as well.

Table 1. Mycotoxins that inhibit protein synthesis and effects.

MYCOTOXINS AND MECHANISMS	**HEALTH EFFECTS**
Trichothecenes: • Inhibit translation of mRNA into protein; highly potent inhibitors of protein synthesis • Lipophylic; easily traverse membranes including lung, capillary and blood brain barrier	Inhibition of Protein Synthesis leads to deficits in • Learning and memory • Autonomic function and other neural function • Change Immune response (antibodies, TNFa, macrophage activity, etc.) resulting in increase in susceptibility to infectious disease and cancer and trigger of autoimmune disorders
Aflatoxins, sterigmatocystin, Ochratoxin A inhibit protein synthesis • At transcription level • At translation level	DNA damage results in • Decrease in antibody production, inhibition of immune defenses, results increased infectious disease, cancer promotion
Tremorgens; paralytic mycotoxins • Inhibit CNS transmitters, e.g., GABA, Glu, Asp	Decrease in inhibitory transmitters results in • Tremors, hyperexcitability, inco-ordination • Paralysis
Gliotoxin • Affects astrocytes • Immune modulator • Apoptosis inducer: increases	Decrease in immune defenses and neural changes • Facilitates infection by *A. fumigatus* • Affects CNS function

caspase-3 activity	

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